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14. ABSTRACT

In cancer, specific growth and invasion-promoting proteins are abnormally over-expressed compared to normal cells and these proteins are often the target of therapies designed to inactivate them. The computational methods developed by our lab was used to identify highly over-expressed genes specifically in cancer cells, a method that was instrumental in identifying the first gene fusion in the majority of prostate cancer, TMPRSS-ETS. Utilizing the same method, the gene SPINK1 was later identified as highly over-expressed in prostate cancer, specifically in prostate cancer patients that were negative for the TMPRSS-ETS gene fusions. An antibody that targets the SPINK1 protein was tested in pre-clinical models for its potential as effective therapy to treat TMPRSS-ETS-negative prostate cancer. Here, an antibody against the SPINK1 protein was used to examine its effects on various prostate cancer cell lines. The anti-SPINK1 antibody was able to inhibit the growth of cells that over-expressed SPINK1 but had no effect on cells that harbored other aberrations. Importantly, the anti-SPINK1 antibody also significantly halted the tumor growth in mice that were implanted with SPINK1 over-expressing tumors. These results suggest that a sub-set of TMPRSS-ETS negative prostate cancer patients that over-express SPINK1 can potentially be successfully treated with anti-SPINK1 antibody.

15. SUBJECT TERMS
TMPRSS-ETS, SPINK1, prostate cancer

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DOD W81XWH-08-1-0031 (Apr 15, 2008 – Apr. 14, 2011)
PI: Arul Chinnaiyan
Final Report

INTRODUCTION:

Our laboratory previously developed a bioinformatics approach termed Cancer Outlier Profile Analysis (COPA) to nominate candidate oncogenes from transcriptomic data based on high expression in a subset of cases (‘‘outlier expression’’) (*1*). Using the Oncomine compendium of tumor profiling studies (http://www.oncomine.org) (*2*), COPA correctly identified several known oncogenes as outliers, such as ERBB2 in breast cancer and PBX1 in leukemia. In addition, COPA also identified the ETS family members ERG and ETV1 as high-ranking outliers in multiple prostate cancer profiling studies, leading to the discovery of recurrent gene fusions involving androgen-regulated gene TMPRSS2 with ERG, ETV1, ETV4, or ETV5 in prostate cancer cases that over-expressed the respective ETS family member (*1*, *3*, *4*). About 40%–80% of prostate-specific antigen (PSA)-screened prostate cancers harbor ETS gene fusion, whereas the remaining cases are driven by other non-fusion molecular aberrations. Additionally, we have determined that ETS-positive and -negative cancers have distinct transcriptional signatures across profiling studies (*5*) suggesting that fusion-negative cancers activate unique set of oncogenes and downstream targets. We used the same outlier meta-analysis approach (meta-COPA) to identify *SPINK1* (serine peptidase inhibitor, Kazal type 1) as a high-ranking meta-outlier in a subset of prostate cancer that was mutually exclusive with ERG and ETV1 outlier expression across multiple prostate cancer profiling studies. SPINK1 encodes a 56–amino acid extracellular secreted peptide and SPINK1 mRNA has been reported to be expressed in various human cancers. Thus, SPINK1 may be an attractive therapeutic target. **Under this study, we have validated the mutual exclusivity of *SPINK1* expression and ETS fusion status, and demonstrated its role in cell proliferation, invasion and tumor growth (11). We also found that *SPINK1* expression can be detected non-invasively in patient urine samples (6, 7) Finally, we demonstrated that *SPINK1*-mediated cell proliferation, invasion and tumor growth can be attenuated by a monoclonal antibody against *SPINK1 (12)*.**

STATEMENT OF WORK

A brief summary of the tasks completed is provided below. Complete details of all experiments can be found in the published manuscripts under “Reportable Outcomes” section.

Task 1: Determine the role of *SPINK1* in prostate cancer cell lines. *Here we propose to over-express SPINK1 in primary prostate epithelial cells and benign immortalized RWPE cells and monitor their phenotype. Similarly using prostate cancer cell lines (i.e., 22Rv1), we plan to knock-down SPINK1 in prostate cancer cell lines the express high levels of SPINK1 (and are TMPRSS2-ETS negative). Various phenotypic readouts will be assessed including cell proliferation, apoptosis, cell invasion/migration, and growth in soft agar.*

We first examined the role of SPINK1 in cell proliferation and invasion in prostate cancer cells. We treated benign immortalized RWPE prostate epithelial cells and DU145 and PC3 prostate

cancer cells (both of which are SPINK1−/ETS−) with recombinant SPINK1, which resulted in a significant increase in cell proliferation (Fig. 1A). We next characterized the effect of rSPINK1 or conditioned media (CM) from 22RV1 cells (a SPINK1+/ETS⁻ cell line) on cell invasion using a Boyden chamber Matrigel invasion assay. As shown in Fig. 1B, addition of rSPINK1 or 22RV1 (prostate cells that overexpress SPINK1) CM to RWPE cells significantly increased invasion. We next demonstrated that the addition of rSPINK1 or 22RV1 CM rescued the invasive phenotype of 22RV1 cells in which SPINK1 was knocked down (Fig. 1C).

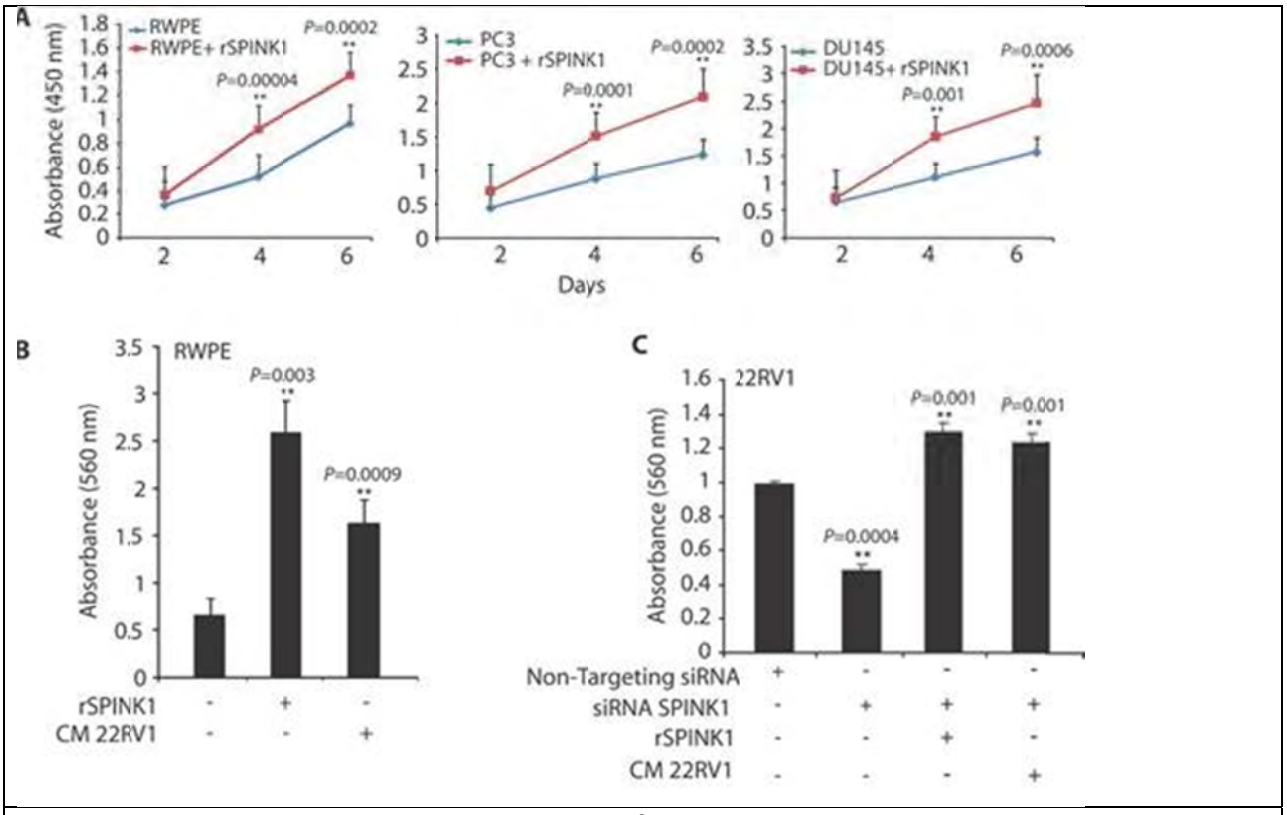

Figure 1. (A) SPINK1 stimulated cell proliferation in SPINK1−/ETS− cell lines. Benign immortalized prostate cell line RWPE and prostate cancer cell lines DU145 and PC3 (all SPINK1−/ETS−) were untreated or treated with rSPINK1 (10 ng/ml). Cell proliferation was measured by a WST-1 colorimetric assay at the indicated time points. **(B)** SPINK1 mediates invasion of RWPE cells as measured by Boyden chamber Matrigel invasion assay. RWPE cells were treated with rSPINK1 (10 ng/ml) or conditioned media (CM) from 22RV1 cells (SPINK1+/ETS−). **(C)** As in (B), except using 22RV1 cells transfected with siRNA against SPINK1. SPINK1-silenced 22RV1 cells were further treated with rSPINK1 (10 ng/ml) or CM from 22RV1 cells.

Next, we investigated the role of SPINK1 in cell invasion and motility in cells where SPINK1 is stably knocked-down. As anticipated, shSPINK1 cells showed decreased cell invasion by more than 75% in a Boyden chamber Matrigel assay compared to nonspecific vector control (shNS) cells (Fig. 2A). Reduction of cell motility in a bead motility assay was also observed in shSPINK1 cells compared to shNS cells (Fig. 2A, top panel).

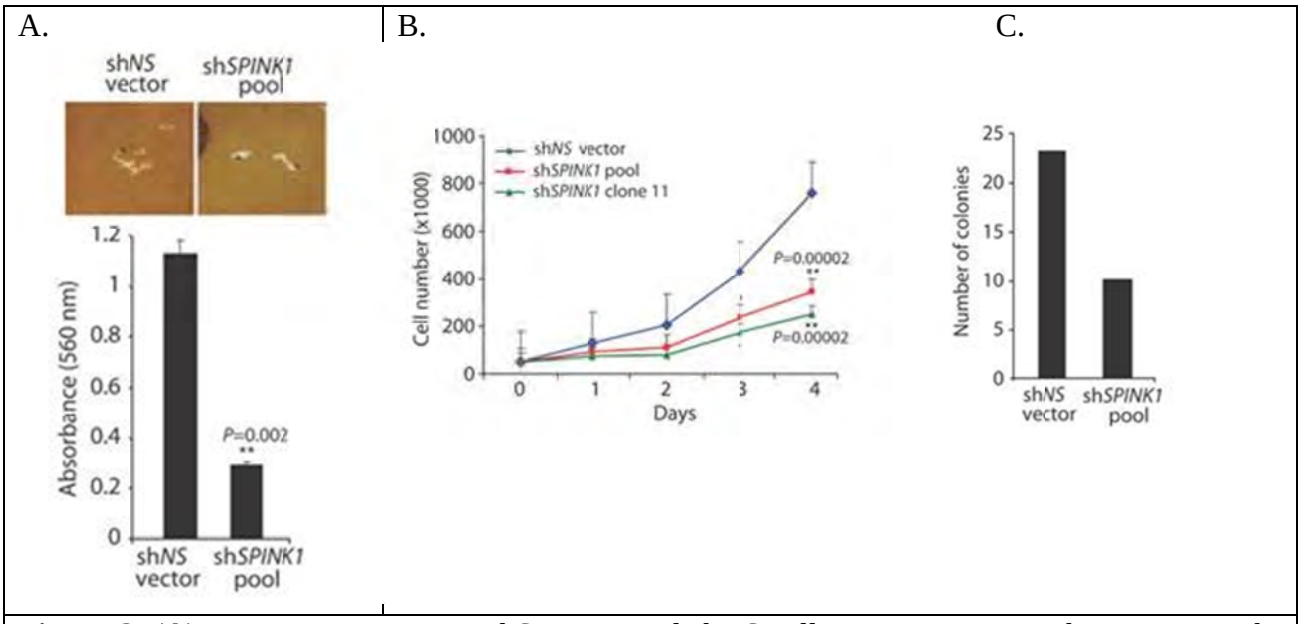

Figure 2. (A) Invasion assay using shSPINK1 and shNS cells. Representative photomicrographs (400× magnification) showing cell motility assay (top inset) are shown. shNS vector cells exhibit longer cell motility tracks compared to shSPINK1 knockdown cells. **(B)** Cell proliferation assay using pooled shSPINK1, shSPINK1 clone 11, or shNS cells at the indicated time points. **(C)** Soft agar colony assay using pooled shSPINK1 and shNS cells. All experiments were independently performed in triplicate. Data represent means ± SEM. P values from significant two-sided Student's t tests are given (*P < 0.05; **P < 0.001).

To investigate the role of SPINK1 in cell proliferation, we carried out assays using pooled shSPINK1, the clone with the greatest SPINK1 knockdown (shSPINK1 clone 11), and shNS cells. Both pooled (55% reduction) and clonal shSPINK1 cells (66% reduction) showed significantly decreased proliferation compared to shNS cells (Fig. 2B). Further, shSPINK1 cells showed decreased soft agar colony formation when compared to shNS cells (Fig. 2C).

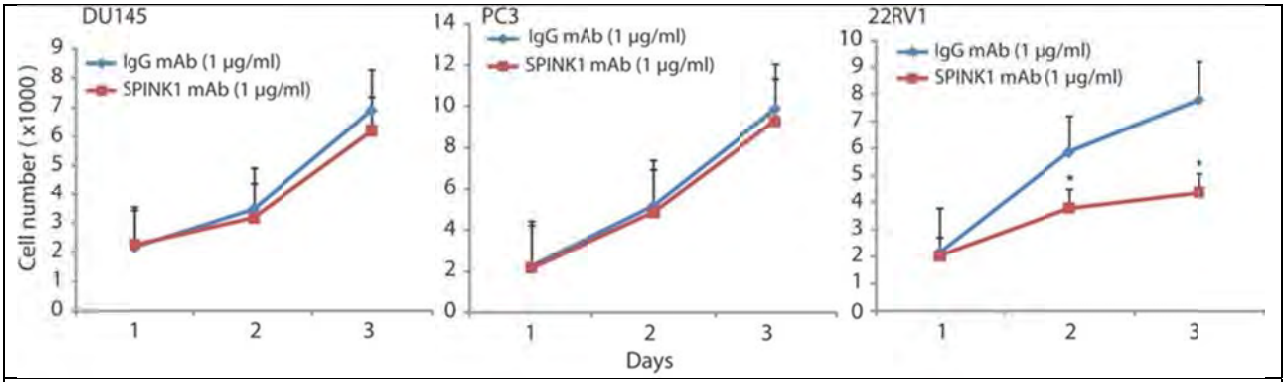

Figure 3. An antibody to SPINK1 attenuates *in vitro* proliferation and invasion exclusively in SPINK1+/ETS− prostate cancer cells. (A) Cell proliferation of DU145, PC3, and 22RV1 cells was assessed in the presence of SPINK1 mAb or IgG mAb (1 µg/ml). Data represent means ± SEM. P values from significant two-sided Student's t tests are given (*P < 0.05; **P < 0.001).

Because our results above demonstrate a role for SPINK1 in invasion and proliferation, and SPINK1 is an extracellular secreted protein, we hypothesized that a monoclonal antibody (mAb) against SPINK1 may be able to directly target SPINK1+/ETS− prostate cancer cells. Thus, we tested the effects of an antibody to SPINK1 on 22RV1 cell proliferation and invasion. The SPINK1 mAb significantly inhibited 22RV1 cell proliferation compared to a control monoclonal immunoglobulin G (IgG) antibody. However, the antibody to SPINK1 had no effect on DU145 and PC3 cell proliferation (Fig. 3).

In addition to inhibiting proliferation, the mAb to SPINK1 (0.5 and 1 μg/ml) significantly attenuated cell invasion by 69 and 81%, respectively, compared to a control IgG mAb in 22RV1 cells (Fig. 4). Similar to 22RV1, which is an androgen signaling–independent derivative of primary CWR22 human prostate xenograft tumors, we also investigated CWR22Pc cells, an androgen signaling–dependent derivative of CWR22, which also express high amounts of SPINK1. As expected, CWR22Pc cell invasion was blocked by 47 and 54% by the mAb to SPINK1 at 0.5 and 1 μg/ml of SPINK1 mAb concentration respectively. However, the mAb to SPINK1 had no significant effect on invasion of SPINK1− prostate cancer cell lines including PC3, DU145, LNCaP, or VCaP (Fig. 4).

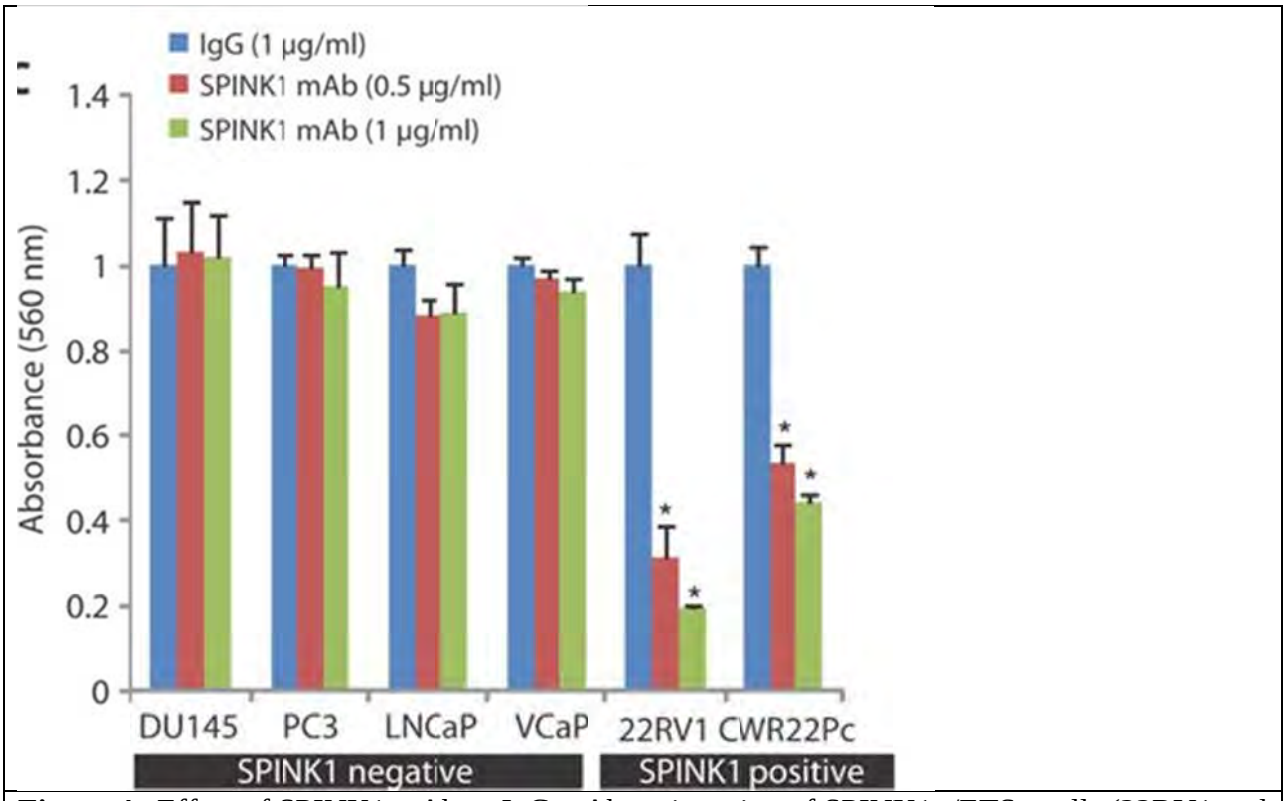

Figure 4. Effect of SPINK1 mAb or IgG mAb on invasion of SPINK1+/ETS− cells (22RV1 and CWR22Pc) and SPINK1-/ETS− cells (DU145, PC3, LNCaP, and VCaP). All experiments were independently performed in triplicates. Data represent means ± SEM. P values from significant two-sided Student's t tests are given (*P < 0.05; **P < 0.001).

Task 2: Determine the mechanism of *SPINK1* over-expression in a subset of prostate cancers. *Here we propose to investigate the potential mechanisms of SPINK1's role in tumor progression in TMPRSS2-ETS negative prostate cancers.*

SPINK1 has a similar structure as EGF, with ~50% sequence homology and three intrachain disulfide bridges (8, 9, 10). We have demonstrated that SPINK1 and EGFR interact in immunoprecipitation assays and furthermore, exogenous SPINK1 is capable of inducing EGFR phosphorylation (12). We next examined the functional consequences of SPINK1-EGFR interaction in the context of SPINK1+ prostate cancer using 22RV1 cells. Transient knockdown of EGFR blocked 22RV1 cell invasion by 75% (Fig. 5A), which was partially rescued by addition of exogenous SPINK1. A similar effect of EGFR knockdown was observed in RWPE cells treated with rSPINK1 (Fig. 5B). These results suggest that some but not all of SPINK1's effects are mediated by EGFR. Because mAbs to EGFR are Food and Drug Administration (FDA)-approved for certain cancers, we sought to determine whether blocking EGFR could inhibit the oncogenic effects of SPINK1. We demonstrated that mAb to EGFR (cetuximab, C225) blocked the cell-invasive effects of rSPINK1 in RWPE cells (Fig. 5C). C225 also blocked cell invasion of SPINK1+ 22RV1 cells but not in SPINK1− cell lines DU145, PC3, LNCaP, or VCaP and combining mAbs to SPINK1 and EGFR had an additive effect in the inhibition of 22RV1 cell invasion (12).

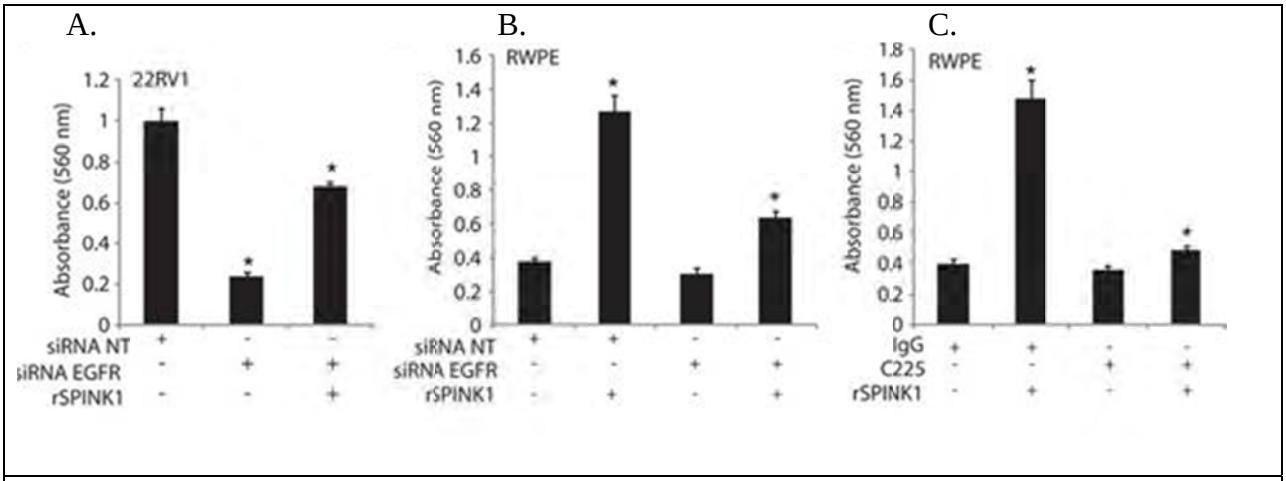

Figure 5. SPINK1 mediates its oncogenic effects in part through EGFR. **(A)** Invasion assay showing siRNA-mediated EGFR knockdown 22RV1 cells treated with rSPINK1 (10 ng/ml). **(B)** Same as in (A), except with RWPE cells. **(C)** Invasion assay showing rSPINK1 (10 ng/ml)–stimulated RWPE cells in the presence or absence of C225 [cetuximab (50 μg/ml)] or IgG mAb (50 μg/ml). All experiments were independently performed in triplicates. Data represent means ± SEM. P values from significant two-sided Student's t tests are given (*P < 0.05; **P < 0.001).

Finally, we investigated the role of SPINK1 *in vivo* as a therapeutic target. To investigate the role of SPINK1 in intravasation, a key step involved in the process of metastasis, we used a chick chorioallantoic membrane (CAM) model system and demonstrated that rSPINK1 induced intravasation of benign RWPE cells (12). Similarly, SPINK1 mAb and C225 significantly inhibited 22RV1 cell intravasation but did not significantly inhibit PC3 cell intravasation (12). To qualify SPINK1 as a potential therapeutic target *in vivo*, we implanted pooled shSPINK1-luciferase (luc) and shNS-luc 22RV1 cells in nude male mice. At both 4 and 5 weeks after

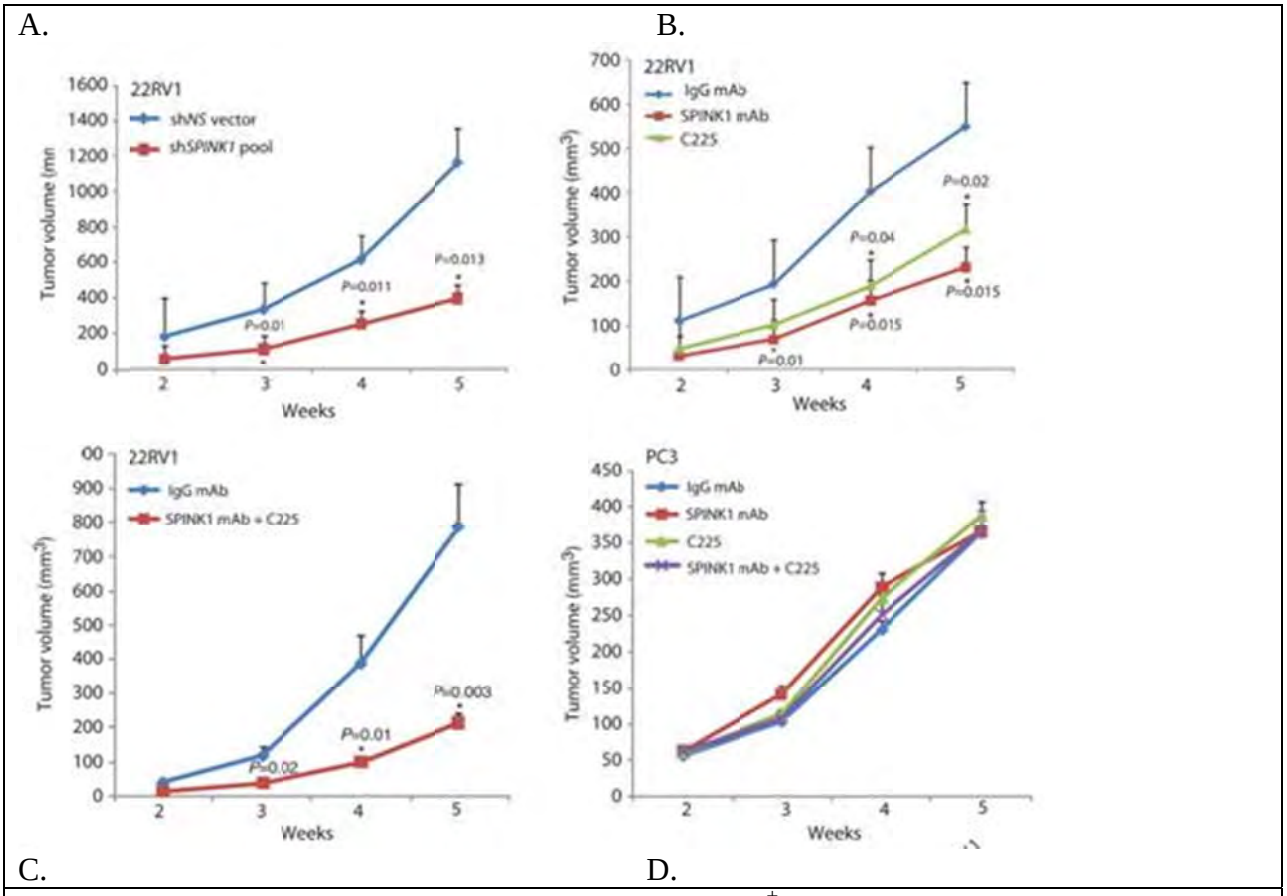

Figure 6. SPINK1 is a therapeutic target in *SPINK1*$^{+}$ prostate cancer. (**A**) Subcutaneous xenograft growth of sh*NS*-luciferase (luc) or sh*SPINK1*-luc 22RV1 cells implanted in male BALB/c nu/nu mice (n = 10 in each group). (**B**) As in (A), except using 22RV1-luc cell xenografts treated with control IgG mAb (n = 8), SPINK1 mAb (n = 6), or C225 (n = 8) (10 mg/kg) twice a week. (**C**) Same as in (B), except mice (n = 7 per group) were treated with a combination of SPINK1 and C225 mAb (10 mg/kg for both). (**D**) As in (B) and (C), except using PC3-luc xenografts treated with control IgG mAb, SPINK1 mAb, or C225 (n = 8 per group) (10 mg/kg) alone or in combination twice a week.

implantation, 22RV1-shSPINK1-luc cells formed significantly smaller tumors (55% reduction at week 4 and 63% reduction at week 5) compared to shNS-luc cells (Fig. 6A).

To demonstrate preclinical efficacy of the mAb to SPINK1, we treated nude mice implanted with 22RV1-luc cells with either the mAb to SPINK1 or an isotype-matched monoclonal IgG twice a week. As shown in Fig. 6B, administration of SPINK1 mAb monotherapy resulted in a 61% reduction of tumor burden at week 4 and 58% reduction at week 5. Because SPINK1 mediates its oncogenic effects in part through EGFR, we similarly assessed the mAb to EGFR (C225) using the same dosage schedule. C225 treatment resulted in a 41% reduction at week 4 and 37% reduction at week 5 (Fig. 6B). By combining mAbs to SPINK1 and EGFR, we observed an additive effect *in vivo* showing a 74 and 73% reduction in the growth of 22RV1 xenografts at weeks 4 and 5 respectively (Fig. 6C).To confirm our *in vitro* results, which suggested no effect of SPINK1 or EGFR inhibition on SPINK1− prostate cancer, we performed a similar xenograft

study using PC3 cells. As expected, neither SPINK1 mAb nor C225 significantly inhibited tumor growth in PC3 xenografted mice (Fig. 6D).

Task 3: Explore the utility of *SPINK1* for the non-invasive detection of prostate cancer in urine biospecimens. *We will examine a cohort of over 400 post-digital rectal exam (post-DRE) urine sediments for outlier expression of SPINK1 by quantitative RT-PCR. We will also correlate to TMPRSS2-ETS expression.*

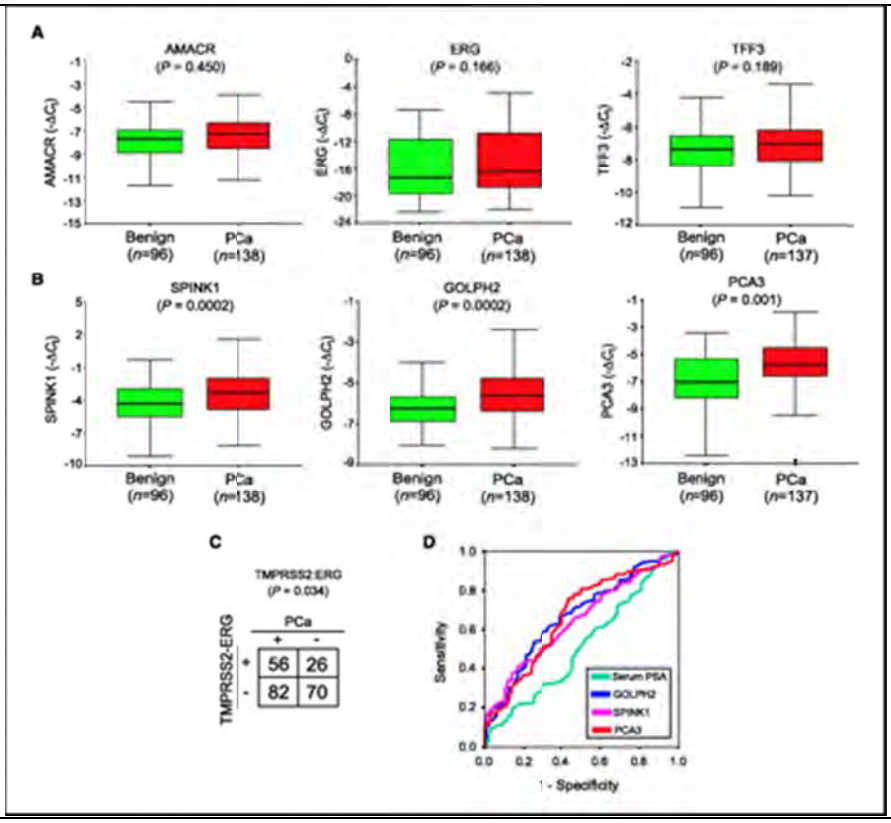

Figure 7. Characterization of candidate urine-based biomarkers of prostate cancer. **A to C**. qPCR was performed on WTA cDNA from urine obtained from patients presenting for needle biopsy or prostatectomy. Biomarker expression in patients with negative needle biopsies (*green*) or patients with prostate cancer (*PCa*; positive needle biopsy or prostatectomy; *red*) is shown. Normalization was performed using $-\Delta C_t$, with *PCA3* normalized to urine *PSA* expression as performed previously. *AMACR, ERG, GOLPH2, SPINK1*, and *TFF3* were normalized to the average of urine sediment *PSA* and *GAPDH* expression. *TMPRSS2:ERG* gene fusion expression was dichotomized as positive or negative. The $-\Delta C_t$ values of genes that were not significant predictors of prostate cancer by univariate analysis are shown in *A*, and the expression of those that were significant predictors is shown in B and C. *P* values from the univariate analysis for the detection of prostate cancer are indicated. **D.** ROC curves for individual variables for the diagnosis of prostate cancer. AUCs for *GOLPH2, PCA3, SPINK1*, and serum PSA are 0.664, 0.661, 0.642, and 0.508, respectively.

Although prostate-specific antigen (PSA) serum level is currently the standard of care for prostate cancer screening in the United States, it lacks ideal specificity and additional biomarkers

are needed to supplement or potentially replace serum PSA testing. We developed a multiplexed qPCR-based test for prostate cancer and assessed seven putative prostate cancer biomarkers, including SPINK1, in sedimented urine on a cohort of patients presenting for biopsy or radical prostatectomy (7). Biomarkers included those generally overexpressed in prostate cancer, such as PCA3, AMACR, and GOLPH2 as well as those overexpressed in subsets of prostate cancers, such as ERG and TMPRSS2:ERG, and TFF3 and SPINK1. All genes were first tested by univariate analysis, with GOLPH2 (P = 0.0002), SPINK1 (P = 0.0002), PCA3 (P = 0.001), and TMPRSS2:ERG fusion (P = 0.034) showing significant association for discriminating patients with prostate cancer from patients with negative needle biopsies (Fig. 7).

Urine-based diagnostic test can detect both TMPRSS:ERG gene fusion as well as other biomarkers of aggressive prostate cancer including SPINK1, in a non-invasive manner. We have also demonstrated that SPINK1 and ETS fusion are mutually exclusive in prostate cancer (11). Therefore we can utilize this assay to differentiate SPINK1 positive tumors from those harboring ETS fusions to treat subtype-specific prostate cancers with appropriate therapies.

KEY RESEARCH ACCOMPLISHMENTS: *Bulleted list of key research accomplishments emanating from this research.*

We have successfully accomplished all of the goals of the proposal and performed additional investigative studies to dissect out the functional role and mechanism of *SPINK1* in the ETS fusion negative prostate cancer. We have demonstrated that:

- ***SPINK1 overexpression promotes cell growth and invasion and knock-down of SPINK1 leads to a decrease in cell growth and invasion***.
- ***SPINK1 shares homology with EGF and SPINK1's effects are mediated partially through interaction with EGFR***.
- ***Treatment with SPINK1 antibody alone or in combination with EGFR antibody reduces growth of SPINK1+ tumors but not SPINK negative tumors.***
- ***SPINK1 can be detected in the urine of prostate cancer patients and may be developed for diagnostic and/or prognostic marker for SPINK1+ prostate cancer.***

REPORTABLE OUTCOMES: *Provide a list of reportable outcomes that have resulted from this research to include: manuscripts, abstracts, presentations; patents and licenses applied for and/or issued; degrees obtained that are supported by this award; development of cell lines, tissue or serum repositories; informatics such as databases and animal models, etc.; funding applied for based on work supported by this award; employment or research opportunities applied for and/or received based on experience/training supported by this award.*

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CONCLUSION: *Summarize the results to include the importance and/or implications of the completed research and when necessary, recommend changes on future work to better address the problem. A "so what section" which evaluates the knowledge as a scientific or medical product shall also be included in the conclusion of the report.*

Utilizing the computational methods developed earlier by our lab that was designed to identify genes that are highly over-expressed specifically in cancer cells, we have shown that *SPINK1* outlier expression defines an aggressive molecular subtype of prostate cancer (~10% of cases). Moreover, SPINK1+ prostate cancers are mutually exclusive for the TMPRSS-ETS gene-fusion (*6*). In this study, we have demonstrated the neoplastic potential of SPINK1 *in vitro* as well as *in vivo.* SPINK1 increased prostate cancer cell proliferation, invasion and tumor growth whereas knock-down of SPINK1 abrogates those oncogenic effects. SPINK1 has 50% sequence homology with EGF and we showed that SPINK1 interacts with EGFR.

SPINK1 is an extracellular secreted protein that may be targetable with a neutralizing antibody. Here, an antibody against the SPINK1 protein was used to examine its effects on various prostate cancer cell lines. The anti-SPINK1 antibody, alone or in combination with EGFR antibody, was able to inhibit the growth of cells that over-expressed SPINK1 but had no effect on cells that harbored other aberrations. Importantly, the anti-SPINK1 antibody also significantly halted the tumor growth in mice that were implanted with SPINK1 over-expressing tumors. These results suggest that a sub-set of TMPRSS-ETS negative prostate cancer patients that over-express SPINK1 can potentially be successfully treated with anti-SPINK1 antibody.

The goals and accomplishments of this proposal have provided the key pre-clinical data that could be instrumental in the diagnosis and treatment of SPINK1+/ETS- prostate cancer patients.

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Therapeutic Targeting of SPINK1-Positive Prostate Cancer
Bushra Ateeq, *et al.*
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Editor's Summary

Targeting Outside the Box

Out-of-the-box thinking is highly valued in all creative endeavors, and science is no exception. Similarly, out-of-the-cell, or extracellular, drug targets have many advantages over intracellular ones, such as easy access by small-molecule inhibitors and antibodies. Because one-third of all cases of prostate cancer—one of the most prevalent forms of the disease in men —are aggressive and fast-growing, and traditional treatments are often unhelpful and cause troublesome side effects, it is clear that some out-of-the-box thinking is required to address this therapeutic dilemma. Now, Ateeq et al. have identified SPINK1 (serine peptidase inhibitor, Kazal type 1) as an extracellular therapeutic target for an aggressive subset of SPINK1+ prostate cancer. SPINK1 is highly expressed in ~10% of prostate cancers, and expression has been correlated with aggressive disease. In the new work, the authors showed directly that SPINK1 contributes to the aggressive phenotype. Forced expression of recombinant SPINK1 increased prostate cancer cell proliferation and invasiveness, whereas knockdown of SPINK1 gene expression or treatment with a SPINK1-directed monoclonal antibody resulted in decreased cell division, invasiveness, and tumor growth. Moreover, SPINK1 mediated its neoplastic effects in part through interactions with the epidermal growth factor receptor (EGFR). Indeed, antibodies to both SPINK1 and EGFR blocked the growth of SPINK1+/ETS – tumors more than either antibody alone and did not affect SPINK1 – tumors. Together, these findings suggest that SPINK1 represents a new, specific, and —by virtue of its outside-of-the-box location—druggable target for a potentially lethal form of prostate cancer.

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CANCER

Therapeutic Targeting of SPINK1-Positive Prostate Cancer

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Gene fusions involving ETS (erythroblastosis virus E26 transformation–specific) family transcription factors are found in ~50% of prostate cancers and as such can be used as a basis for the molecular subclassification of prostate cancer. Previously, we showed that marked overexpression of *SPINK1* (*serine peptidase inhibitor, Kazal type 1*), which encodes a secreted serine protease inhibitor, defines an aggressive molecular subtype of ETS fusion–negative prostate cancers (*SPINK1*$^+$/*ETS*$^-$, ~10% of all prostate cancers). Here, we examined the potential of SPINK1 as an extracellular therapeutic target in prostate cancer. Recombinant SPINK1 protein (rSPINK1) stimulated cell proliferation in benign RWPE as well as cancerous prostate cells. Indeed, RWPE cells treated with either rSPINK1 or conditioned medium from 22RV1 prostate cancer cells (*SPINK1*$^+$/*ETS*$^-$) significantly increased cell invasion and intravasation when compared with untreated cells. In contrast, knockdown of *SPINK1* in 22RV1 cells inhibited cell proliferation, cell invasion, and tumor growth in xenograft assays. 22RV1 cell proliferation, invasion, and intravasation were attenuated by a monoclonal antibody (mAb) to SPINK1 as well. We also demonstrated that SPINK1 partially mediated its neoplastic effects through interaction with the epidermal growth factor receptor (EGFR). Administration of antibodies to SPINK1 or EGFR (cetuximab) in mice bearing 22RV1 xenografts attenuated tumor growth by more than 60 and 40%, respectively, or ~75% when combined, without affecting PC3 xenograft (*SPINK1*$^-$/*ETS*$^-$) growth. Thus, this study suggests that SPINK1 may be a therapeutic target in a subset of patients with *SPINK1*$^+$/*ETS*$^-$ prostate cancer. Our results provide a rationale for both the development of humanized mAbs to SPINK1 and evaluation of EGFR inhibition in *SPINK1*$^+$/*ETS*$^-$ prostate cancers.

INTRODUCTION

Therapies targeted against specific molecular alterations present only in cancer cells have revolutionized the treatment of several cancers. For example, targeting ERBB2, which is amplified in ~20% of breast cancers, with the humanized monoclonal antibody (mAb) trastuzumab (Herceptin) has resulted in improved survival for breast cancer patients. Although organ-confined prostate cancer is highly curable, more than 32,000 U.S. men are expected to die of metastatic prostate cancer in 2010 (*1*). Multiple approved therapies (and newer agents in late-stage development) target the androgen signaling axis in metastatic disease; however, additional targeted therapies are lacking.

We previously used a bioinformatics approach, cancer outlier profile analysis (COPA), to systematically prioritize genes with marked overexpression in a subset of cancers (outlier expression). This strategy identified outlier expression of the ETS (erythroblastosis virus E26 transformation–specific) family members *ERG* and *ETV1* in a subset of prostate cancers across multiple gene expression profiling studies. It also led to the discovery of recurrent gene fusions involving the 5′ untranslated region of the androgen-regulated gene *TMPRSS2* with ETS transcription factors (*ERG*, *ETV1*, *ETV4*, or *ETV5*) (*2*–*5*). Subsequent in vitro and in vivo studies have demonstrated a driving role for ETS fusions in prostate oncogenesis and cancer progression (*6*–*9*).

Subsequently, we used a "meta-outlier approach," which used COPA to prioritize genes that consistently showed high-ranking outlier expression across multiple profiling studies. This approach identified *SPINK1* (*serine peptidase inhibitor, Kazal type 1*) as a high-ranking meta-outlier in a subset of prostate cancer with mutually exclusive outlier expression of *ERG* and *ETV1* across multiple prostate cancer profiling studies (*10*). *SPINK1*, also known as *pancreatic secretory trypsin inhibitor* (*PSTI*) or *tumor-associated trypsin inhibitor* (*TATI*), encodes a 56–amino acid peptide thought to protect the pancreas from autodigestion by preventing premature activation of pancreatic proteases (*11*). Apart from its normal expression in pancreatic acinar cells, *SPINK1* mRNA has been reported to be expressed in various human cancers (*12*–*18*), and increased serum SPINK1 concentration has been correlated with poor prognosis in some studies (*12*, *13*, *17*). The prostate gland also secretes a variety of serine proteases, most notably the kallikrein enzyme PSA (prostate-specific antigen), but also trypsin (*19*). Thus, SPINK1 may have a role in modulating the activity of cancer-related proteases in other tissues besides the pancreas.

We confirmed the mutually exclusive overexpression of SPINK1 and *ETS* gene fusions using a combined immunohistochemistry (for SPINK1) and fluorescence in situ hybridization (FISH) (for *ETS* fusions) approach across multiple independent cohorts, and demonstrated that *SPINK1* outlier expression is associated with an aggressive subset of prostate cancers (*10*). We also demonstrated that *SPINK1* outlier expression can be detected noninvasively in urine and con-

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tributes to a multiplexed panel of biomarkers, which outperforms serum PSA for prostate cancer diagnosis in patients presenting for needle biopsy (*10*, *20*). Our combined analyses of more than 1500 prostate cancer cases demonstrated *SPINK1* outlier expression in ~10% of all PSA-screened prostate cancers, which were invariably negative for *ETS* gene fusions (*SPINK1*$^+$/*ETS*$^-$) (*10*). Furthermore, *SPINK1*$^+$ tumors show shorter PSA recurrence-free survival in prostatectomy-treated patients (*10*) and shorter progression-free survival in endocrine-treated patients (*21*).

Unlike *ETS* gene fusions that lead to the overexpression of a transcription factor (which are difficult to target therapeutically), *SPINK1* encodes an extracellular secreted protein and thus is potentially more amenable to therapeutic targeting. Here, we qualify SPINK1 as a therapeutic target in *SPINK1*$^+$/*ETS*$^-$ prostate cancer and demonstrate the therapeutic potential of a mAb to SPINK1 in preclinical models. Additionally, we demonstrate that SPINK1 mediates its oncogenic effects in part through epidermal growth factor receptor (EGFR) and that a mAb to EGFR shows in vitro and in vivo activity in *SPINK1*$^+$ prostate cancer.

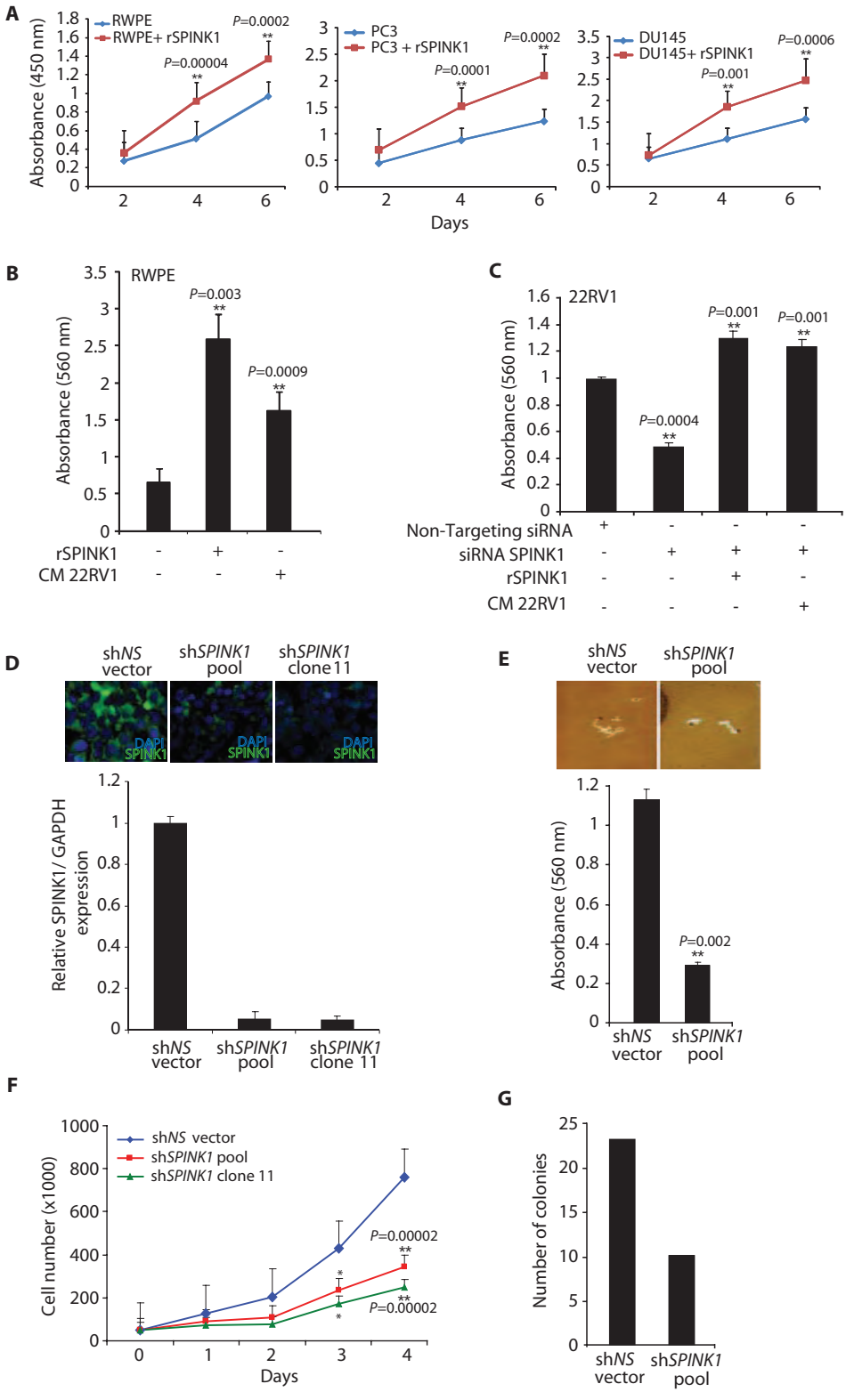

Fig. 1. *SPINK1* has oncogenic effects in prostate cells in vitro. (**A**) SPINK1 stimulated cell proliferation in *SPINK1*$^-$/*ETS*$^-$ cell lines. Benign immortalized prostate cell line RWPE and prostate cancer cell lines DU145 and PC3 (all *SPINK1*$^-$/*ETS*$^-$) were untreated or treated with rSPINK1 (10 ng/ml). Cell proliferation was measured by a WST-1 colorimetric assay at the indicated time points. (**B**) SPINK1 mediates invasion of RWPE cells as measured by Boyden chamber Matrigel invasion assay. RWPE cells were treated with rSPINK1 (10 ng/ml) or conditioned media (CM) from 22RV1 cells (*SPINK1*$^+$/*ETS*$^-$). (**C**) As in (B), except using 22RV1 cells transfected with siRNA against *SPINK1*. *SPINK1*-silenced 22RV1 cells were further treated with rSPINK1 (10 ng/ml) or CM from 22RV1 cells. (**D**) *SPINK1* expression in *SPINK1* knockdown 22RV1 cells (stable pooled sh*SPINK1* or stable sh*SPINK1* clone 11) compared to nontargeting pooled stable control (sh*NS* vector) cells by qPCR (transcript) or immunofluorescence using an antibody against SPINK1 (protein, upper inset; 600× magnification). (**E**) Invasion assay using sh*SPINK1* and sh*NS* cells. Representative photomicrographs (400× magnification) showing cell motility assay (top inset) are shown. sh*NS* vector cells exhibit longer cell motility tracks compared to sh*SPINK1* knockdown cells. (**F**) Cell proliferation assay using pooled sh*SPINK1*, sh*SPINK1* clone 11, or sh*NS* cells at the indicated time points. (**G**) Soft agar colony assay using pooled sh*SPINK1* and sh*NS* cells. All experiments were independently performed in triplicate. Data represent means ± SEM. *P* values from significant two-sided Student's *t* tests are given (*$P < 0.05$; **$P < 0.001$).

RESULTS

SPINK1 as an autocrine factor in prostate cancer

To further investigate the role of *SPINK1* in prostate cancer, we determined the effects of exogenous SPINK1 on invasion and proliferation using recombinant hexahistidine (6XHis)–tagged SPINK1 protein (rSPINK1) (fig. S1A) or conditioned media (CM) collected from 22RV1 prostate cancer cells (*SPINK1*$^+$/*ETS*$^-$) (fig. S1B) (*10*). We treated benign immortalized RWPE prostate epithelial cells and DU145 and PC3 prostate cancer cells (both of which are *SPINK1*$^-$/*ETS*$^-$) with rSPINK1 (10 ng/ml), which resulted in a significant increase in cell proliferation (Fig. 1A). We next characterized the effect of rSPINK1 or 22RV1 CM on cell invasion using a Boyden chamber Matrigel invasion assay. As shown in Fig. 1B, addition of rSPINK1 or 22RV1 CM to RWPE cells significantly increased invasion (P = 0.003 and 0.0009, respectively). Similar effects were observed when MCF7 breast cancer cells were treated with rSPINK1 or 22RV1 CM (fig. S1C). Multiple recombinant 6XHis-tagged control proteins or CM collected from RWPE or LNCaP prostate cancer cells did not induce invasion in RWPE cells (figs. S1D and S2).

We previously showed that transient small interfering RNA (siRNA)–mediated knockdown of *SPINK1* in 22RV1 cells decreased cell invasion (*10*). Here, we extended these results by demonstrating that the addition of rSPINK1 or 22RV1 CM rescued the invasive phenotype of 22RV1 cells in which *SPINK1* was knocked down (Fig. 1C; P = 0.001 for both rSPINK1 and 22RV1 CM).

We next investigated whether the exogenous effect of SPINK1 on cell proliferation and invasion was dependent on protease inhibitory activity of trypsin [which has been shown to be simultaneously expressed with SPINK1 in different tumor types (*17*, *22*)] or PSA. Initial experiments demonstrated that *PRSS1* (trypsinogen) mRNA expression in 22RV1 cells is relatively low compared with the CAPAN-1 pancreatic cancer cell line (fig. S3A), although a significant increase in *PRSS1* transcript was observed in siRNA-mediated *SPINK1* knockdown 22RV1 cells (fig. S3B). However, as shown in fig. S3C, stimulation of 22RV1 cells with rSPINK1 or EGF did not affect trypsin expression. siRNA-mediated knockdown of *PRSS1* in 22RV1 cells also had no effect on invasion (fig. S3, D and E). Similarly, stimulation of 22RV1 cells with rSPINK1 or EGF did not significantly affect PSA expression (fig. S4A). Finally, blocking PSA with a mAb did not significantly inhibit 22RV1 cell invasion (fig. S4B). Together, these findings demonstrate that extracellular SPINK1 induces prostate cancer cell proliferation and invasion independent of protease inhibitory activity of trypsin or PSA. Although effects on other proteases cannot be excluded, our results suggest that SPINK1 is an autocrine pro-proliferative and proinvasive factor with effects independent of trypsin and PSA activity.

The role of SPINK1 in cell proliferation and invasion

To further investigate the role of *SPINK1* in cell proliferation and invasion, we generated short hairpin RNA (shRNA) against *SPINK1* and established stable 22RV1 cells where SPINK1 was silenced (sh*SPINK1*). Knockdown of *SPINK1* in both pooled and clonal sh*SPINK1* cells compared to nontargeting control cells (sh*NS* cells) was confirmed at the RNA level by quantitative polymerase chain reaction (qPCR) (more than 80% in both), as well as at the protein level by immunofluorescence staining with an antibody against SPINK1 (Fig. 1D). Next, we investigated the role of *SPINK1* in cell invasion and motility using sh*SPINK1* cells. As anticipated, sh*SPINK1* cells showed decreased cell invasion by more than 75% in a Boyden chamber Matrigel assay compared to nonspecific vector control (sh*NS*) cells (Fig. 1E; P = 0.002). Reduction of cell motility in a bead motility assay was also observed in sh*SPINK1* cells compared to sh*NS* cells (Fig. 1E, top panel).

To investigate the role of *SPINK1* in cell proliferation, we carried out assays using pooled sh*SPINK1*, the clone with the greatest *SPINK1* knockdown (sh*SPINK1* clone 11), and sh*NS* cells. Both pooled (55% reduction) and clonal sh*SPINK1* cells (66% reduction) showed significantly decreased proliferation compared to sh*NS* cells (Fig. 1F; P = 0.00002 in both cases). Further, sh*SPINK1* cells showed decreased soft agar colony formation when compared to sh*NS* cells (Fig. 1G).

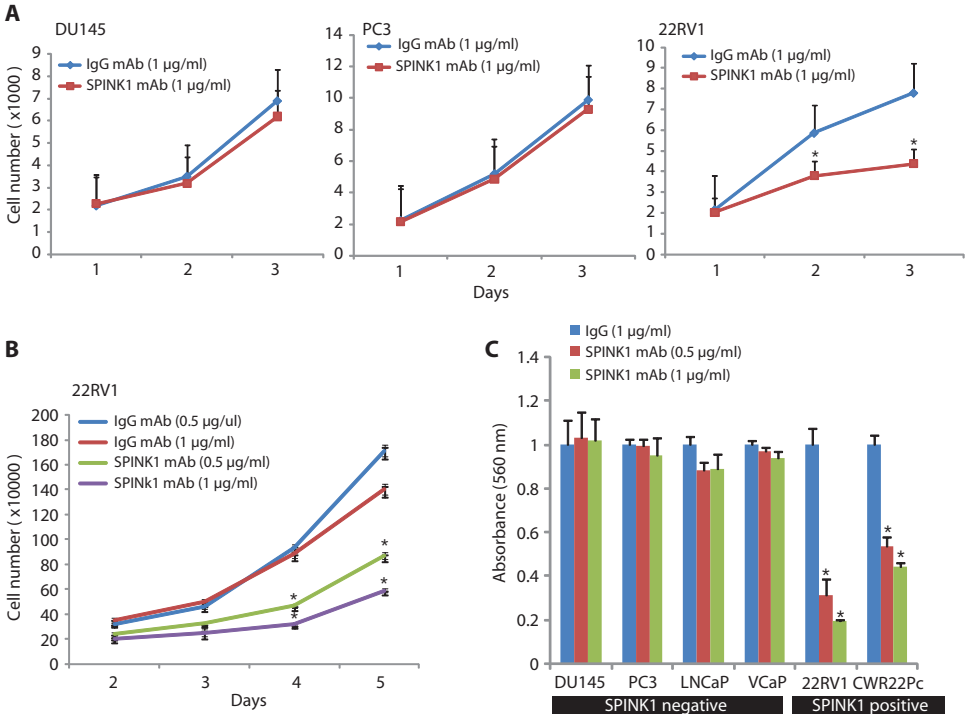

Fig. 2. An antibody to SPINK1 attenuates in vitro proliferation and invasion exclusively in *SPINK1*$^+$/*ETS*$^-$ prostate cancer cells. (**A**) Cell proliferation of DU145, PC3, and 22RV1 cells was assessed in the presence of SPINK1 mAb or IgG mAb (1 μg/ml). (**B**) As in (A), except using 22RV1 cells and SPINK1 mAb or IgG mAb (0.5 to 1 μg/ml). (**C**) Effect of SPINK1 mAb or IgG mAb on invasion of *SPINK1*$^+$/*ETS*$^-$ cells (22RV1 and CWR22Pc) and *SPINK1*$^+$/*ETS*$^-$ cells (DU145, PC3, LNCaP, and VCaP). All experiments were independently performed in triplicates. Data represent means ± SEM. P values from significant two-sided Student's t tests are given (*P < 0.05; **P < 0.001).

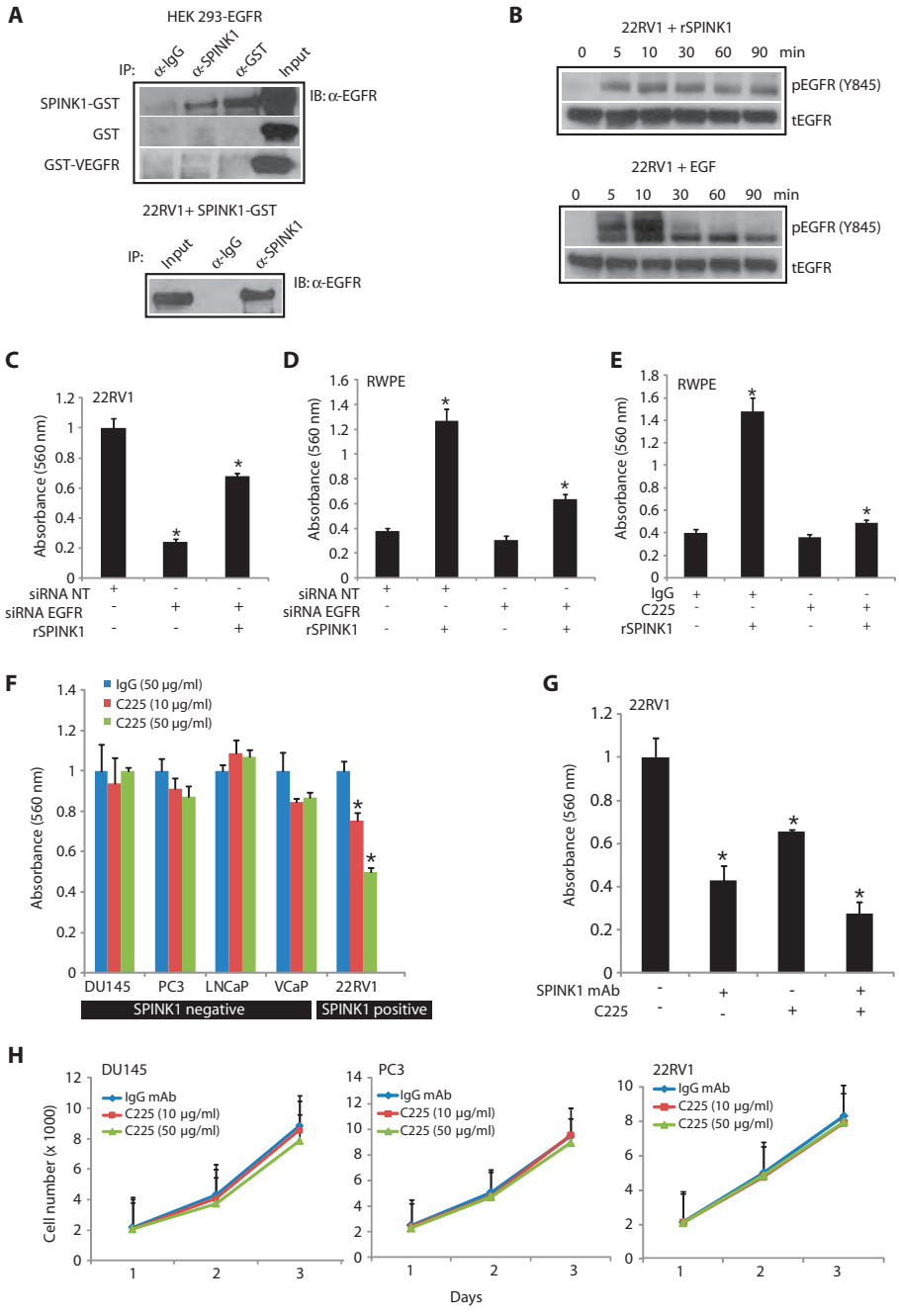

Fig. 3. SPINK1 mediates its oncogenic effects in part through EGFR. (**A**) Immunoprecipitation using antibodies to IgG, SPINK1, or GST of exogenous SPINK1-GST, GST, or GST-VEGFR added to HEK 293 cells transfected with EGFR and immunoblotted with an antibody to EGFR (top panel), and immunoprecipitation using antibodies to IgG or SPINK1 of exogenous SPINK1-GST added to 22RV1 cells and immunoblotted with an antibody to EGFR (bottom panel). (**B**) Western blot showing EGFR phosphorylation in response to rSPINK1 (100 ng/ml) or EGF (10 ng/ml) stimulation. (**C**) Invasion assay showing siRNA-mediated *EGFR* knockdown 22RV1 cells treated with rSPINK1 (10 ng/ml). (**D**) Same as in (C), except with RWPE cells. (**E**) Invasion assay showing rSPINK1 (10 ng/ml)–stimulated RWPE cells in the presence or absence of C225 [cetuximab (50 μg/ml)] or IgG mAb (50 μg/ml). (**F**) Invasion assay showing the effect of IgG or C225 antibody on *SPINK1*$^+$ and *SPINK1*$^-$ cancer cells. (**G**) As in (F), except 22RV1 cells were treated with a combination of antibodies to SPINK1 (1 μg/ml) and/or C225 (50 μg/ml). (**H**) Cell proliferation assay using the indicated cells in the presence of IgG mAb or C225. All experiments were independently performed in triplicates. Data represent means ± SEM. *P* values from significant two-sided Student's *t* tests are given (*$P < 0.05$; **$P < 0.001$).

In vitro targeting of SPINK1 using a mAb

Because our results above demonstrate a role for *SPINK1* in invasion and proliferation, and SPINK1 is an extracellular secreted protein, we hypothesized that a mAb against SPINK1 may be able to directly target *SPINK1*$^+$/*ETS*$^-$ prostate cancer cells. Thus, we tested the effects of an antibody to SPINK1 on 22RV1 cell proliferation and invasion. The SPINK1 mAb (0.5 and 1 μg/ml) significantly inhibited 22RV1 cell proliferation by 40 and 50%, respectively, compared to a control monoclonal immunoglobulin G (IgG) antibody (Fig. 2, A and B; $P = 0.0001$ and $P = 0.0007$, respectively). However, the antibody to SPINK1 had no effect on DU145 and PC3 cell proliferation.

In addition to inhibiting proliferation, the mAb to SPINK1 (0.5 and 1 μg/ml) significantly attenuated cell invasion by 69 and 81%, respectively, compared to a control IgG mAb in 22RV1 cells (Fig. 2C; $P = 0.002$ and $P = 0.007$, respectively). Similar to 22RV1, which is an androgen signaling–independent derivative of primary CWR22 human prostate xenograft tumors, we also investigated CWR22Pc cells, an androgen signaling–dependent derivative of CWR22 (*23*), which also express high amounts of SPINK1. As expected, CWR22Pc cell invasion was blocked by 47 and 54% by the mAb to SPINK1 at 0.5 and 1 μg/ml of SPINK1 mAb concentration (Fig. 2C; $P = 0.003$ and $P = 0.002$, respectively). The mAb to SPINK1 had no significant effect on invasion of *SPINK1*$^-$ prostate cancer cell lines including PC3, DU145, LNCaP, or VCaP (Fig. 2C). Finally, the mAb to SPINK1 attenuated 22RV1 cell motility compared to IgG control, but had no effect on PC3 (*SPINK1*$^-$/*ETS*$^-$) cell motility (fig. S5A).

Oncogenic effects of SPINK1 in part through interaction with EGFR

SPINK1 has a similar structure as EGF, with ~50% sequence homology and three intrachain disulfide bridges (*24*, *25*). To characterize potential SPINK1 and EGFR interaction, we overexpressed EGFR in human embryonic kidney (HEK) 293 cells and incubated the lysates with SPINK1-GST (glutathione *S*-transferase), GST, or GST-VEGF (vascular endothelial growth factor) receptor 2 (GST-VEGFR) recom-

binant proteins. We observed a strong interaction between SPINK1-GST and EGFR but not with GST alone or GST-VEGFR recombinant protein (Fig. 3A, top panel). Endogenous SPINK1 and EGFR interaction was not detected by immunoprecipitation and immunoblotting in 22RV1 cells, because of the secretory nature of the SPINK1 protein. However, addition of GST-SPINK1 to 22RV1 cells followed by immunoprecipitation and immunoblotting confirmed the interaction of SPINK1 and endogenous EGFR in 22RV1 cells (Fig. 3A, bottom panel).

To further delineate the role of EGFR mediation of SPINK1 in prostate cancer, we next assessed whether exogenous SPINK1 was capable of inducing EGFR phosphorylation (similar to the cognate ligand EGF). Stimulating 22RV1 cells with rSPINK1 resulted in EGFR phosphorylation, although weaker than that observed with EGF (Fig. 3B). rSPINK1 stimulation resulted in sustained EGFR phosphorylation over a 90-min time course, whereas EGF resulted in strong EGFR phosphorylation, which diminished after only 10 min. Similarly, stable sh*SPINK1* knockdown 22RV1 cells (pooled and clonal) showed decreased phosphorylated EGFR (pEGFR), with slightly decreased total EGFR (possibly because of EGFR degradation) (fig. S6A). Finally, we demonstrate that rSPINK1 is able to induce dimerization of EGFR, although more weakly than EGF (fig. S6B).

We next examined the functional consequences of SPINK1-EGFR interaction in the context of *SPINK1*$^+$ prostate cancer using 22RV1 cells. Transient knockdown of EGFR (fig. S5B) blocked 22RV1 cell invasion by 75% (Fig. 3C; $P = 0.004$), which was partially rescued by addition of exogenous SPINK1. A similar effect of EGFR knockdown was observed in RWPE cells treated with rSPINK1 (Fig. 3D; $P = 0.014$ and $P = 0.021$, respectively). These results suggest that some but not all of SPINK1's effects are mediated by EGFR.

Because mAbs to EGFR are Food and Drug Administration (FDA)–approved for certain cancers, we sought to determine whether EGFR blockade could inhibit the oncogenic effects of SPINK1. We first demonstrated that mAb to EGFR (cetuximab, C225) blocked the cell-invasive effects of rSPINK1 in RWPE cells (Fig. 3E). C225 also blocked cell invasion of *SPINK1*$^+$ 22RV1 cells but not in *SPINK1*$^-$ cell lines DU145, PC3, LNCaP, or VCaP (Fig. 3F). Combining mAbs to SPINK1 and EGFR had an additive effect in the inhibition of 22RV1 cell invasion (Fig. 3G; $P = 0.001$). In contrast to mAb to SPINK1 (Fig. 2A), C225 had no effect on 22RV1 cell proliferation or PC3 and DU145 cell proliferation (Fig. 3H). Together, these experiments suggest that SPINK1 has both EGFR-dependent and EGFR-independent functions in prostate cancer.

As a preliminary exploration of the downstream signaling pathways involved in the SPINK1-EGFR axis, we studied the mitogen-activated protein kinase (MAPK) and protein kinase B/AKT pathways in stable *SPINK1* knockdown 22RV1 cells (sh*SPINK1* clone 11). We observed decreased pMEK (phosphorylated mitogen-activated or extracellular signal–regulated protein kinase kinase), pERK (phosphorylated extracellular signal–regulated kinase), and pAKT (phosphorylated AKT) in stable sh*SPINK1* cells compared to control sh*NS* cells (fig. S5C). Likewise, 22RV1 cells treated with SPINK1 mAb antibody showed decreased pERK (fig. S5D). These observations provide the foundation for further studies of the SPINK1-EGFR axis.

The role of SPINK1 in vivo and as a therapeutic target

Our in vitro studies demonstrated that SPINK1 mediates cell proliferation and invasion in *SPINK1*$^+$ prostate cancer cells, and suggested that a mAb can target extracellular SPINK1. To investigate the role of *SPINK1* in intravasation, a key step involved in the process of metastasis, we used a chick chorioallantoic membrane (CAM) model system (*26*) and demonstrate that rSPINK1 induced intravasation of benign RWPE cells (Fig. 4A). Similarly, SPINK1 mAb and C225 significantly inhibited 22RV1 cell intravasation ($P = 0.01$ and $P = 0.03$, respectively), but did not significantly inhibit PC3 cell intravasation (Fig. 4, B and C).

To qualify SPINK1 as a potential therapeutic target in vivo, we implanted pooled sh*SPINK1*-luciferase (luc) and sh*NS*-luc 22RV1 cells in nude male mice. At both 4 and 5 weeks after implantation, 22RV1-sh*SPINK1*-luc cells formed significantly smaller tumors (55% reduction at week 4, $P = 0.008$, and 63% reduction at week 5, $P = 0.013$) compared to sh*NS*-luc cells (Fig. 4, D and H).

To demonstrate preclinical efficacy of the mAb to SPINK1, we treated nude mice implanted with 22RV1-luc cells with either the mAb to SPINK1 or an isotype-matched monoclonal IgG (10 mg/kg) twice a week. As shown in Fig. 4, E and I, administration of SPINK1 mAb monotherapy resulted in a 61% reduction of tumor burden at week 4 ($P = 0.015$) and 58% reduction at week 5 ($P = 0.015$). A significant decrease in Ki-67–positive immunostained nuclei was observed in the SPINK1 mAb–treated group compared to the control group (fig. S7).

Because SPINK1 mediates its oncogenic effects in part through EGFR, we similarly assessed the mAb to EGFR (C225) using the same dosage schedule. C225 treatment resulted in a 41% reduction at week 4 ($P = 0.04$) and 37% reduction at week 5 ($P = 0.02$) (Fig. 4, E and I). By combining mAbs to SPINK1 and EGFR, we observed an additive effect in vivo showing a 74 and 73% reduction in the growth of 22RV1 xenografts at weeks 4 ($P = 0.01$) and 5 ($P = 0.003$), respectively (Fig. 4, F and I).

To confirm our in vitro results, which suggested no effect of SPINK1 or EGFR inhibition on *SPINK1*$^-$ prostate cancer, we performed a similar xenograft study using PC3 cells. As expected, neither SPINK1 mAb nor C225 significantly inhibited tumor growth in PC3 xenografted mice (Fig. 4, G and I). Finally, to investigate the potential toxicity of SPINK1 mAb therapy, we investigated whether the mAb to SPINK1 interacts with SPINK3, the murine homolog of SPINK1. The mAb to SPINK1 used in our studies does not recognize murine SPINK3, thus explaining the lack of observed toxicity in SPINK1 mAb–treated mice (fig. S8, A to C).

DISCUSSION

Previous studies demonstrated that *SPINK1* outlier expression identified a subset of *ETS*-negative prostate cancers (~10% of all PSA-screened prostate cancers), although the mechanism for SPINK1 outlier expression remains unknown (*10*). *SPINK1* defines a distinct molecular subtype of prostate cancer characterized by lack of *ETS* gene fusions as well as a more aggressive phenotype as corroborated by independent groups across distinct cohorts of prostate cancer patients (*10*, *21*). Thus, our working hypothesis is that *SPINK1*$^+$ prostate cancer represents an aggressive form of prostate cancer that may respond to different therapies than *ETS* gene fusion–positive prostate cancers.

Here, we show that *SPINK1* promotes prostate cancer proliferation and invasion through autocrine and paracrine signaling. We also demonstrate an in vivo role for SPINK1 in intravasation and tumor xeno-

graft growth. At present, the precise mechanism and signaling pathways responsible for these effects in *SPINK1*$^+$ prostate cancer are unclear. A recent study showed that mutation of *SPINK1* at leucine 18 (L18) in the trypsin interaction site reduced tumor growth, angiogenesis, and lung metastases in HT-29 5M21 human colon carcinoma tumor xenografts, suggesting that the cancer-related phenotypes of *SPINK1* may be related to its anti-proteinase activity (*27*). Moreover, the invasive behavior of these HT-29 5M21 colon cancer cells was abolished with an antibody to SPINK1 (*27*). However, in our study, we did not observe any effect of SPINK1 on trypsin or PSA, two candidate proteases in prostate cancer.

Recent studies also indicate that *SPINK1* may be an apoptosis inhibitor prevent-

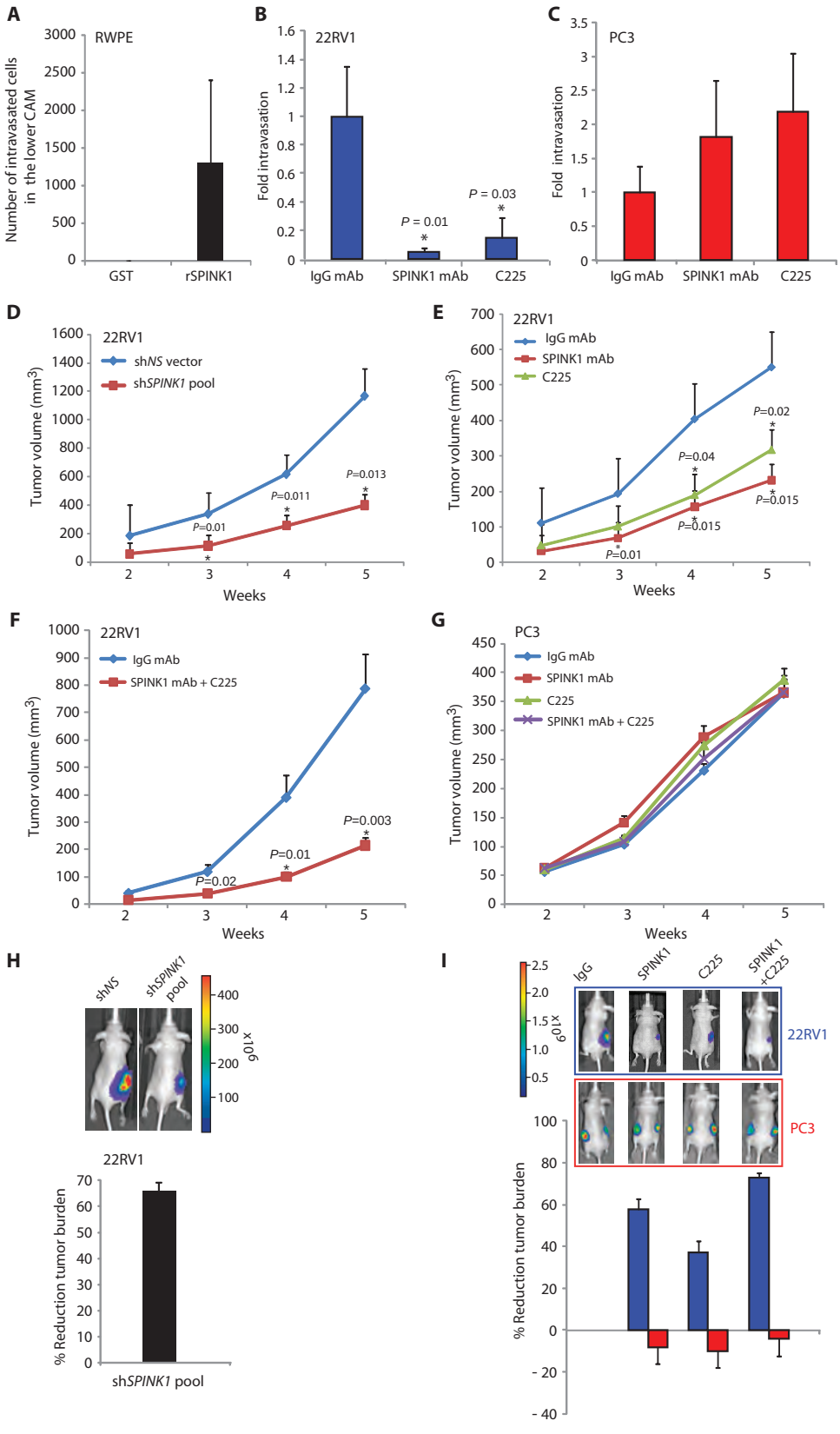

Fig. 4. SPINK1 is a therapeutic target in *SPINK1*$^+$ prostate cancer. (**A**) Chick chorioallantoic membrane (CAM) assay quantifying intravasated RWPE cells upon stimulation with rSPINK1 ($n = 6$ in each group). (**B**) CAM assay using 22RV1 cells in the presence of IgG mAb, SPINK1 mAb, or C225 ($n = 5$ in each group), with fold change of intravasated cells compared to IgG mAb plotted. (**C**) As in (B), except using PC3 cells. (**D**) Subcutaneous xenograft growth of sh*NS*-luciferase (luc) or sh*SPINK1*-luc 22RV1 cells implanted in male BALB/c nu/nu mice ($n = 10$ in each group). (**E**) As in (D), except using 22RV1-luc cell xenografts treated with control IgG mAb ($n = 8$), SPINK1 mAb ($n = 6$), or C225 ($n = 8$) (10 mg/kg) twice a week. (**F**) Same as in (E), except mice ($n = 7$ per group) were treated with a combination of SPINK1 and C225 mAb (10 mg/kg for both). (**G**) As in (E) and (F), except using PC3-luc xenografts treated with control IgG mAb, SPINK1 mAb, or C225 ($n = 8$ per group) (10 mg/kg) alone or in combination twice a week. (**H**) Representative bioluminescence images from mice in (D) bearing pooled sh*NS*-luc or sh*SPINK1*-luc xenografts and percent reduction in tumor volume at week 5. (**I**) Same as (H), except bioluminescence images from mice bearing 22RV1-luc xenografts (red, top panel) or PC3-luc (blue, lower panel) mice treated with IgG mAb, SPINK1 mAb, or C225 mAb alone or in combination, with comparative percent reduction plot in tumor volume at week 5. Data represent means ± SEM. *P* values from significant two-sided Student's *t* tests are given (*$P < 0.05$; **$P < 0.001$).

ing serine protease-dependent cell death (*28*). Here, we show that SPINK1, which has structural similarities with EGF (*29*), binds to EGFR, and inhibiting SPINK1 attenuates key downstream mediators of the EGFR pathway including MEK, ERK, and AKT. Furthermore, we also show that SPINK1 dimerizes EGFR and induces sustained phosphorylation of EGFR, which have been shown to be critical for downstream signaling activation after ligand binding (*30*). However, in contrast to SPINK1 mAb, EGFR mAb only partially inhibited the cell-invasive effects of 22RV1 cells and had no effect on cell proliferation, suggesting that SPINK1 engages both EGFR-dependent and EGFR-independent pathways to mediate its oncogenic effects. SPINK1 has also been shown to engage the EGFR/MAPK cascade in NIH 3T3 fibroblasts and pancreatic cancer cells (*31*).

This study provides compelling evidence that *SPINK1* overexpression is oncogenic in prostate cancer and that inhibition of *SPINK1* via RNA interference or blocking antibodies may have therapeutic potential. Our preclinical models suggest that this therapeutic effect would only be effective in patients with *SPINK1*$^+$ prostate cancer, suggesting that such therapies would need to be evaluated in a molecularly guided fashion. Because the area of antibody-based therapeutics for extracellular targets is well developed, based on examples such as trastuzumab in breast cancers with ERBB2 overexpression, we postulate that a SPINK1-blocking antibody may have similar efficacy on a molecularly defined subset of prostate cancers. We have previously demonstrated that patients with the subset of *SPINK1*$^+$/*ETS*$^-$ prostate cancers can be reliably identified by immunohistochemistry (*10*, *20*), as would be required for a molecularly defined clinical trial. Although humanized SPINK1 mAbs are not yet available for clinical testing, our studies show that SPINK1 partially mediates its oncogenic effects through EGFR.

This finding prompted us to evaluate the utility of the FDA-approved EGFR mAb cetuximab, which showed in vitro and in vivo activity only against *SPINK1*$^+$ prostate cancer cells (although less effective than SPINK1 mAb). Phase I/II clinical trials of cetuximab (*32*) and EGFR small molecules have been largely disappointing in metastatic prostate cancer (*33*, *34*); however, a small subset of patients have had responses, including 3 of 36 (8%) patients who showed >50% PSA decline in a Phase Ib/IIa clinical trial of cetuximab in combination with doxorubicin in castrate-resistant metastatic prostate cancer patients (*32*). Results from our study provide a plausible mechanism for why only the limited subset of patients with positive cancers (~10% of all cases) may benefit from EGFR inhibition. This hypothesis can be assessed retrospectively and in biomarker-informed clinical trials of patients with *SPINK1*$^+$ prostate cancer. Because the mAb to SPINK1 used in our studies did not interact with murine SPINK3 (the homolog of SPINK1), our study does not inform on the potential toxicity of SPINK1 mAb therapy. However, an FDA-approved mAb to EGFR has specific in vivo activity against *SPINK1*$^+$ prostate cancer, providing an immediately translatable strategy for targeting *SPINK1*$^+$ cancers that can be clinically investigated while toxicity of humanized SPINK1 antibody therapy is explored.

In summary, our results support *SPINK1* as an oncogene in a subset of prostate cancers that can be molecularly identified, and provide the rationale to develop humanized SPINK1 antibodies for human clinical trials. Our work also reinforces the molecular subclassification of prostate cancer in clinical trials (whether through *SPINK/ETS* status or other relevant biomarkers), which has lagged behind other common epithelial cancers (that is, breast, lung, and colon).

MATERIALS AND METHODS

Cell lines and *SPINK1* knockdown

The benign immortalized prostate cell line RWPE as well as prostate cancer cell lines DU145, PC3, and 22RV1 were obtained from the American Type Culture Collection (ATCC) and were grown according to ATCC guidelines. For stable knockdown of *SPINK1*, human lentiviral shRNAmir individual clone (ID V2LHS_153419) targeting against *SPINK1* or nonsilencing lentiviral shRNAmir in GIPZ vectors was purchased from Open Biosystems (Thermo Scientific Open Biosystems). Details are available in Supplementary Materials and Methods.

Quantitative PCR

Total RNA was isolated with a miRNeasy mini kit following the manufacturer's instruction (Qiagen). Complementary DNA was synthesized from 1 μg of total RNA with SuperScript III (Invitrogen) in the presence of random primers. qPCR was performed with the StepOne Real-Time PCR system (Applied Biosystems). Details and primer information are available in Supplementary Materials and Methods.

Cell proliferation assay

Proliferation for control and experimental cells was measured by a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases (cell proliferation reagent WST-1; Roche Diagnostics) at the indicated time points in triplicate. Cell counts for sh*NS* vector and sh*SPINK1* cells were estimated by trypsinizing cells and analysis by Coulter counter (Beckman Coulter) at different time points in triplicates.

Basement membrane matrix invasion assay

For invasion assays, sh*NS* vector– or sh*SPINK1*-transduced cells, as well as RWPE, PC3, and 22RV1 cells were used. Equal numbers of the indicated cells were seeded onto the basement membrane matrix (BD Biosciences) present in the insert of a 24-well culture plate. RPMI media supplemented with 10% fetal bovine serum were added to the lower chamber as a chemoattractant. After 48 hours, noninvading cells and extracellular matrix were removed with a cotton swab. Invaded cells were stained with crystal violet and photographed. The inserts were treated with 10% acetic acid, and absorbance was measured at 560 nm.

CAM assay

The assay was performed essentially as described (*26*). Two million RWPE cells were mixed with either 200 ng of multiple tag control protein or 200 ng of rSPINK1 protein and applied to the CAM of 11-day-old chicken embryo. Similarly, 2 million 22RV1 or PC3 cells were mixed with either monoclonal IgG or antibodies to SPINK1 or C225 (1 μg/ml) and applied onto the upper CAM of a fertilized chicken embryo. Three days after implantation, the relative number of cells that intravasate into the vasculature of the lower CAM was analyzed by extracting genomic DNA with the Puregene DNA purification system. Quantification of the human cells in the extracted DNA was done as described (*35*).

22RV1 and PC3 xenograft models

Four-week-old male BALB/c nu/nu mice were purchased from Charles River Inc. (Charles River Laboratory). Stable 22RV1 sh*NS*-luc and 22RV1

sh*SPINK1*-luc cells (5×10^5), or 22RV1-luc (2×10^5) or PC3-luc (5×10^5) cells were resuspended in 100 μl of saline with 20% Matrigel (BD Biosciences) and were implanted subcutaneously into the left flank regions of the mice. Details are available in Supplementary Materials and Methods.

Statistical analysis

All values presented in the study were expressed as means ± SEM. The significant differences between the groups were analyzed by a Student's *t* test, and a *P* value of <0.05 or <0.001 was considered significant.

SUPPLEMENTARY MATERIAL

www.sciencetranslationalmedicine.org/cgi/content/full/3/72/72ra17/DC1
Materials and Methods
Fig. S1. rSPINK1 or CM collected from 22RV1 cells induces invasion in benign or cancer cells.
Fig. S2. CM collected from 22RV1 cells induces cell invasion, but not CM, from LNCaP cells.
Fig. S3. *PRSS1* (trypsin1) knockdown in 22RV1 cells has no effect on SPINK1-mediated cell invasion.
Fig. S4. Exogenous rSPINK1 has no effect on PSA in 22RV1 cells.
Fig. S5. SPINK1 mAb reduces SPINK1$^+$ cell motility and SPINK1 knockdown alters MAPK pathway.
Fig. S6. Exogenous SPINK1 induces EGFR dimerization and phosphorylation.
Fig. S7. SPINK1 mAb induces decrease in tumor proliferation index.
Fig. S8. Anti-SPINK1 mAb, which does not recognize the murine homolog of SPINK1 (SPINK3), has no observed toxic effect in treated mice.
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